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(54) Title: METHODS AND THERAPEUTIC COMPOSITIONS FOR TREATING CYSTIC FIBROSIS

(57) Abstract

A method for generating cystic fibrosis transmembrane conductance regulator (CFTR) function in cells containing mutant CFTR and methods for treating cystic fibrosis are described. The method for generating CFTR function in cells containing mutant CFTR involves contacting the cell with an agent which increases the level of mutant CFTR at cellular locations where wild-type CFTR normally functions such that the mutant CFTR generates functional ion channels or mediates ion transport at the cellular locations where wild-type CFTR normally functions. The methods for treating cystic fibrosis involve administering to a subject the above-described agent. Other aspects described include therapeutic compositions and packaged drugs.

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METHODS AND THERAPEUTIC COMPOSITIONS FOR TREATING CYSTIC FIBROSIS

Related Applications

This application is a continuation-in-part application of USSN 08/072, 708, filed June 7, 1993, which is a continuation-in-part of USSN 07/935,603, filed August 26, 1992, which is a continuation-in-part of USSN 07/613,592, filed November 15, 1990, which is in turn a continuation-in-part application of USSN 07/589,295, filed September 27, 1990, which is a continuation-in-part application of USSN 07/488,307, filed March 5, 1990. This application is also related to the subject matter described in co-pending application USSN 07/985,478 filed December 2, 1992. The contents of all of the above co-pending patent applications are incorporated herein by reference. Definitions of language or terms not provided in the present application are the same as those set forth in the copending applications. Any reagents or materials used in the examples of the present application whose source is not expressly identified also is the same as those described in the copending application, e.g., ΔF508 CFTR gene and CFTR antibodies.

Background of the Invention

Cystic Fibrosis (CF) is the most common fatal genetic disease in humans (Boat, T. et al. Cystic fibrosis. In: The Metabolic Basis of Inherited Disease, C. Scriver, A. Beaudet, W. Sly, and D. Valle, eds. (McGraw Hill, New York, 1989), 2649-2860). Based on both genetic and molecular analysis, a gene associated with CF was isolated as part of 21 individual cDNA clones and its protein product predicted (Kerem, B-S. et al. *Science* 245:1073-1080 (1989); Riordan, J. et al. *Science* 245:1066-1073 (1989); Rommens, J.H. et al. *Science* 245:1059-1065 (1989).

USSN 488,307 describes the construction of the gene into a continuous strand, expression of the gene as a functional protein and confirmation that mutations of the gene are responsible for CF. (*See also* Gregory, R.J. et al. *Nature* 347:382-386 (1990); Rich, D.P. et al. *Nature* 347:358-363 (1990)). The copending patent application also discloses experiments which showed that proteins expressed from wild type but not a mutant version of the cDNA complemented the defect in the cAMP regulated chloride channel shown previously to be characteristic of CF.

The protein product of the CF associated gene is called the cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan, J. et al. *Science* 245:1066-1073 (1989)). CFTR is a protein of approximately 1480 amino acids made up of two repeated elements, each having six transmembrane segments and a nucleotide binding domain. The two repeats are separated by a large, polar, so-called R-domain containing multiple potential phosphorylation sites. Based on its predicted domain structure, CFTR is a member of a class of related proteins which includes the multi-drug resistance (MDR) or P-glycoprotein, bovine

adenyl cyclase, the yeast STE6 protein as well as several bacterial amino acid transport proteins (Riordan, J. et al. *Science* 245:1066-1073 (1989); Hyde, S.C. et al. *Nature* 346:362-365 (1990). Proteins in this group, characteristically, are involved in pumping molecules into or out of cells.

CFTR has been postulated to regulate the outward flow of anions from epithelial cells in response to phosphorylation by cyclic AMP-dependent protein kinase or protein kinase C (Riordan, J. et al. *Science* 245:1066-1073 (1989); Frizzell, R.A. et al. *Science* 233:558-560 (1986); Welsh, M.J. and Liedtke, C.M. *Nature* 322:467 (1986); Li, M. et al. *Nature* 331:358-360 (1988); Hwang, T-C. et al. *Science* 244;1351-1353 (1989); Li, M. et al. *Science* 244:1353-1356 (1989)).

Sequence analysis of the CFTR gene of CF chromosomes has revealed a variety of mutations (Cutting, G.R. et al. *Nature* 346:366-369 (1990); Dean, M. et al. *Cell* 61:863-870 (1990); Kerem, B-S. et al. *Science* 245:1073-1080 (1989); Kerem, B-S. et al. *Proc. Natl. Acad. Sci.* USA 82:488-492 (1990)). Population studies have indicated that the most common CF mutation, a deletion of the three nucleotides that encode phenylalanine at position 508 of the CFTR amino acid sequence (ΔF508), is associated with approximately 70% of the cases of cystic fibrosis. This mutation results in the failure of an epithelial cell chloride channel to respond to cAMP (Frizzell, R.A. et al. *Science* 233:558-560 (1986); Welsh, M.J. *Science* 232:1648-1650 (1986); Li, M. et al. *Nature* 331:358-360 (1988); Quinton, P.M. *Clin. Chem.* 35:726-730 (1989)). In airway cells, this leads to an imbalance in ion and fluid transport. It is widely believed that this causes abnormal mucus secretion, and ultimately results in pulmonary infection and epithelial cell damage.

Studies on the biosynthesis (Cheng, S.H. et al., *Cell* 63:827:834 (1990); Gregory, R.J. et al., *Mol. & Cell Biol.* 11:3886-3893 (1991)) and localization (Denning, G.M. et al., *J. Cell Biol.* 118:551-559 (1992)) of CFTR ΔF508, as well as other CFTR mutants, indicate that many CFTR mutant proteins are not processed correctly and, as a result, are not delivered to the plasma membrane (Gregory, R.J. et al., *Mol. & Cell Biol.* 11:3886-3893 (1991)). These conclusions are consistent with earlier functional studies which failed to detect cAMP stimulated Cl^- channels in cells expressing CFTR ΔF508 (Rich, D.P. et al., *Nature* 347:358-363 (1990); Anderson, M.P. et al., *Science* 251:679-682 (1991)).

Summary of Invention

The present invention is based, at least in part, on the discovery that exposure of cells containing mutant CFTR to various agents, such as differentiating agents, protein enhancing agents, mobilizing agents, and carboxy-compounds, which increase the level of mutant CFTR at cellular locations where wild-type CFTR normally functions, results in CFTR function in the cell. The agents of the present invention facilitate the delivery of mutant CFTR to the appropriate location within the cell and the mutant CFTR of the cells so

treated is capable of generating functional ion channels, e.g. chloride channels, or mediating ion transport.

Butyrate is a well known differentiating agent. Butyrate also is a protein enhancing agent in that the addition of butyrate to the cells containing mutant CFTR results in an increased level of mutant CFTR within the cell. The increased level of mutant CFTR can lead to the passage of the mutant CFTR by the surveillance of the quality control mechanism present in the endoplasmic reticulum of the cell allowing the delivery of the mutant CFTR to the plasma membrane.

Gases, such as air, at a temperature below 37°C, are mobilizing agents. Exposure of cells containing mutant CFTR to mobilizing agents, such as air at a temperature below 37°C, results in a shift in the glycosylation pattern of mutant CFTR towards the form of mutant CFTR which localizes at cellular locations where wild-type CFTR normally functions, such as the plasma membrane.

The present invention pertains to a method of generating CFTR function in a cell containing mutant CFTR. The method involves contacting the cell with an agent, such as a differentiating agent, a protein enhancing agent, a mobilizing agent, or a carboxy-compound, which increases the level of mutant CFTR at cellular locations where wild-type CFTR normally functions such that the mutant CFTR generates functional ion channels at the cellular locations where wild-type CFTR normally functions.

The present invention further pertains to a method of redistributing the subcellular locations of mutant CFTR in a cell containing mutant CFTR. The method involves contacting the cell with an agent which increases the level of mutant CFTR at cellular membranes across which wild-type CFTR mediates ion transport such that the mutant CFTR mediates ion transport across the cellular membranes where wild-type CFTR normally mediates ion transport.

The present invention still further pertains to a method for treating a subject having cystic fibrosis (CF). The method involves the administration of an effective amount of an agent which increases the level of mutant CFTR at cellular locations where wild-type CFTR normally functions such that the mutant CFTR generates functional ion channels or mediates ion transport at the cellular locations where wild-type CFTR normally functions.

The present invention also pertains to a method for treating a subject's airway epithelia containing a mutant CFTR. The method involves contacting a subject's airway epithelia with an agent which increases the level of the mutant CFTR at the plasma membrane such that the mutant CFTR mediates chloride ion transport across the plasma membrane.

Other aspects of the present invention include therapeutic compositions and packaged drugs for treating subjects having CF. The therapeutic compositions include a therapeutically effective amount of at least one of the aforementioned agents, (protein enhancing agent, differentiating agent or carboxy-compound) and a pharmaceutically

acceptable carrier. The packaged drug includes at least one of the aforementioned agents and instructions for administrating the agent for treating subjects having CF.

Brief Description of the Drawings

Figure 1 is a graph depicting the effect of butyrate on recombinant C127-CFTRΔF508 cells.

Figure 2 is a graph depicting the effect of butyrate on ΔF508 airway epithelial cells.

Figure 3 depicts a graph showing the effect of temperature on the processing of CFTR ΔF508 and wild-type CFTR.

Detailed Description

The present invention pertains to a method for generating cystic fibrosis transmembrane conductance regulator (CFTR) function in a cell containing mutant CFTR. The method involves contacting the cell containing the mutant CFTR with an agent which increases the level of the mutant CFTR at cellular locations where wild-type CFTR normally functions such that the mutant CFTR generates functional ion channels at the cellular locations where wild-type CFTR normally functions.

The language "cystic fibrosis transmembrane conductance regulator function" is intended to include functions normally performed by wild-type CFTR. Such functions can include mediation of ion, e.g. chloride ion, transport across cellular membranes, such as the plasma membrane.

The language "wild-type cystic fibrosis transmembrane conductance regulator (CFTR)" and "mutant cystic fibrosis transmembrane conductance regulator (CFTR)" are intended to include wild-type cystic fibrosis transmembrane conductance regulator and mutant cystic fibrosis transmembrane conductance regulator. The sequences of both the DNA encoding the wild-type regulator and the DNA encoding the mutant regulator were described previously in the copending applications identified under the Related Applications section. Mutant CFTRs include proteins having mutations introduced at residues known to be altered in CF chromosomes (ΔG508, ΔI507, R334W, S5491, G551D) and at residues believed to play an important role in the function of CFTR (e.g., K464N, F508R, N894, 900Q, K1250M). *See* Example 7 of USSN 935,603, filed August 26, 1992.

The language "cell containing mutant cystic fibrosis transmembrane conductance regulator" is intended to include a cell which, as it exists in nature, contains mutant CFTR or a cell which, as it exists in nature, does not contain mutant CFTR but is engineered to express mutant CFTR. Examples of cells that contain mutant CFTR as they exist in nature include airway epithelial cells such as nasal and lung epithelia of CF victims. Examples of cells which do not normally contain mutant CFTR but can be engineered to express mutant CFTR include 3T3 fibroblasts, C127, and COS-7 cells. These cells can be

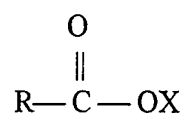
transfected with nucleic acid which encodes and directs expression of mutant CFTR, such as CFTRΔF508. *See* Example 15 of USSN 935,603, filed August 26, 1992.

The language "an agent which increases the level of the mutant cystic fibrosis transmembrane conductance regulator at cellular locations where wild-type cystic fibrosis transmembrane conductance regulator normally functions" is intended to include compounds which increase the level of mutant CFTR at cellular locations where wild-type CFTR normally functions over the level of mutant CFTR at cellular locations where wild-type CFTR normally functions prior to treatment with the agent. The agents of the present invention can increase the level of mutant CFTR at cellular locations where wild-type CFTR normally functions by any mechanism as long as the result is an increased level of mutant CFTR at cellular locations where wild-type CFTR normally functions such that the mutant CFTR generates functional ion channels or mediates ion transport at the cellular location(s) where wild-type CFTR normally functions. It is further contemplated that agents of the present invention can increase the level of functional ion channels, such as chloride ion channels, other than CFTR at cellular locations where wild-type CFTR normally functions. The presence of these functional ion channels in increased amounts at the cellular locations where wild-type CFTR normally functions can at least partially compensate for deficient ion transport in cystic fibrosis victims. The agents of the present invention can increase the level of functional ion channels other than CFTR at cellular locations where wild-type CFTR normally functions by increasing the intracellular level of functional ion channels other than CFTR over that level within the cell prior to treatment with an agent of the present invention. Alternatively, an agent of the present invention can alter the subcellular distribution of an ion channel other than CFTR such that there is a shift toward the form of the channel that localizes at cellular locations where wild-type CFTR normally functions. The agents of the present invention can increase the level of functional ion channels other than CFTR at cellular locations where wild-type CFTR normally functions by promoting differentiation of a cell associated with cystic fibrosis. The agents of the present invention can increase the activity of functional ion channels other than CFTR.

The term "agent" includes protein enhancing agents. For example, some agents of the present invention are capable of increasing the intracellular level of cellular protein over that level within the cell prior to treatment with the respective agent. The mode for measuring this increase is not an important aspect of this invention as long as it provides a means for comparing the pre-and post-treatment levels. The cellular protein which is increased can be any protein present within the cell. The cellular protein which is increased preferably is CFTR or mutant CFTR. The protein enhancing agent can increase the expression of the protein by any mechanism as long as the end result is an increased level of at least one cellular protein. For example, the protein enhancing agent can enhance transcription and/or translation. Examples of protein enhancing agents include carboxylates, carboxylic acids, transcription factors (e.g., AP1, PU.1) and proto-oncogenes which enhance

transcription. An example of a specific protein enhancing agent is butyrate, particularly sodium butyrate.

Preferred protein enhancing agents of the present invention are those having the following formula:



(I)

wherein R has one to five carbon atoms and is a moiety selected from the group consisting of alkyl, alkenyl, and alkynyl; and X is hydrogen or a pharmaceutically acceptable salt.

The alkyl, alkenyl, and alkynyl groups (hereinafter "hydrocarbon groups") can be straight or branched chain moieties. The unsaturated groups can have a single site of unsaturation or a plurality of sites of unsaturation. The hydrocarbon groups can contain up to about five carbon atoms, more preferably up to about four carbon atoms, most preferably up to about three carbon atoms. Examples of hydrocarbon groups which can be used in the present invention include methyl, ethyl, ethenyl, ethynyl, propyl, propenyl, propynyl, butyl, butyenyl, butynyl. Examples of branched chain groups include isobutyl and isopropyl. The protein enhancing agents of the present invention can be purchased or alternatively can be synthesized using conventional techniques. For example, butyrate, particularly sodium butyrate is commercially available.

The language "pharmaceutically acceptable salt" is art-recognized terminology. Typically these salts are capable of being hydrolyzed or solvated under physiological conditions. Examples of such salts include sodium, potassium, and hemisulfate. The term further is intended to include lower hydrocarbon groups capable of being hydrolyzed or solvated under physiological conditions, i.e. groups which esterify the carboxyl group, e.g. methyl, ethyl, and propyl.

The protein enhancing agents of the present invention can be purchased or alternatively can be synthesized using conventional techniques. For example, butyrate, particularly sodium butryate, is commercially available.

The term "agent" also includes mobilizing agents. The mobilizing agents of the present invention are capable of altering the subcellular distribution of mutant forms of CFTR such that there is a shift toward the form of mutant CFTR which localizes at cellular locations where wild-type CFTR normally functions. Wild-type CFTR expressed in 3T3 fibroblasts at 37°C is present in three forms: band A is unglycosylated protein; band B is core glycosylated protein, which is sensitive to endoglycosidase H, a feature associated with processing in the endoplasmic reticulum; and band C is mature CFTR, which is more extensively glycosylated, a characteristic of processing in the Golgi complex. In contrast, some mutant forms of CFTR, such as CFTRΔF508, expressed in cells at 37°C are present

predominantly in bands A and B, suggesting that the proteins do not reach the Golgi complex. Cheng, S.H. et al. *Cell* 83:827-834 (1990). However, it has been surprisingly found that when cells containing mutant CFTR, which is expressed in cells predominantly in bands other than the band representing the glycosylation pattern of mature form of mutant CFTR, come in contact with a mobilizing agents, such as a gas at a temperature below 37°C, there is a shift in the glycosylation pattern towards that of the mature form. The influence of the mobilizing agent on the processing of mutant CFTR is further confirmed by pulse-chase experiments which show that mutant protein synthesized at 37°C is processed normally during subsequent incubation at 26°C but not at 37°C. Furthermore, as confirmed by whole-cell patch clamp studies (conducted at 35°C) on cAMP-activated currents in the plasma membrane of cells expressing CFTR ΔF508, cells containing mutant CFTR, when exposed to a mobilizing agent, deliver mutant CFTR to the plasma membrane. *See* USSN 935,603, the contents of which were expressly incoporated by reference. Examples of such mobilizing agents include gases, such as air, at a temperature below 37°C. Preferred temperatures of the mobilizing agent range from about 10°C to about 35°C. More preferably the temperature of the mobilizing agent ranges from about 20°C to about 35°C. Other mobilizing agents include non-toxic, non-immunogenic agents that, when implanted in the vicinity of the lung, lower the temperature of the lung.

Other agents of the present invention capable of increasing the level of mutant CFTR at cellular locations where wild-type CFTR normally functions include differentiating agents. The language "differentiating agent" is intended to include an agent capable of inducing the differentiation of a cell. Examples of such agents include retinoic acid, other TPA (phorbol esters), DMSO, DMF, interleukins, and proto-oncogenes capable of inducing differentiation.

Still other agents of the present invention capable of increasing the level of mutant CFTR at cellular locations where wild-type CFTR regulator normally functions include carboxy compounds. The language "carboxy-compound" is intended to include compounds which contain a carboxy group ( ). Examples of subgenuses of carboxy-compounds include carboxylic acids and carboxylates. Examples of species of carboxy-compounds include butyric acid and butyrate. The preferred carboxy-compounds are those of formula (I) described above.

The language "cellular locations where wild-type cystic fibrosis transmembrane conductance regulator normally functions" is intended to include cellular locations where wild-type CFTR normally resides when it performs its normal cellular functions. Such cellular locations include the plasma membrane of the cell.

The present invention further pertains to a method for redistributing subcellular locations of mutant CFTR in a cell containing mutant CFTR. The method involves contacting the cell containing the mutant CFTR with an agent which increases the level of the mutant CFTR at cellular membranes across which wild-type CFTR normally

mediates ion transport such that the mutant CFTR mediates ion transport across the cellular membranes which wild-type CFTR mediates ion transport.

The language "redistributing subcellular locations of mutant cystic fibrosis transmembrane conductance regulator" is intended to include relocalization of mutant CFTR from one subcellular location to another. For example, some forms of mutant CFTR accumulate in the endoplasmic reticulum rather than traveling through the Golgi complex to the plasma membrane of the cell containing the mutant form of CFTR. Cheng, S.H. et al. *Cell* 63:827-834 (1990). The agents of the present invention, when in contact with the cell containing the mutant CFTR, promote the relocalization of mutant CFTR from cellular locations where wild-type CFTR does not normally function to cellular locations where wild-type CFTR normally functions. All other terms are as described above.

The present invention even further pertains to a method for treating a subject having cystic fibrosis. The method involves administration of an agent which increases the level of mutant CFTR at cellular locations where wild-type CFTR normally functions such that the mutant CFTR generates functional ion channels and/or mediates ion transport at the cellular locations where wild-type CFTR normally functions.

The term "subject" is intended to include living organisms susceptible to CF, e.g., mammals. Examples of subjects include humans, dogs, cats, horses, cows, goats, rats and mice. The term subject furthers is intended to include transgenic species.

Cystic fibrosis (CF) is a well-known disease state. CF is a disease of infants, children, adolescents, and young adults involving the exocrine glands, especially those secreting mucus. Symptoms associated with CF include pancreatic insufficiency, chronic pulmonary disease, abnormally high sweat electrolyte levels, and, in some cases, cirrhosis of the liver.

The agent of the present invention can be administered to a subject through a route of administration which allows the agent (e.g., protein enhancing agent, mobilizing agent) to perform its intended function, e.g., increasing the level of mutant CFTR at cellular locations where wild-type CFTR normally functions. Examples of routes of administration which can be used include injection (subcutaneous, intravenous, parenterally, intraperitoneally, etc.), oral, inhalation (e.g. for the gaseous mobilizing agents), transdermal, and rectal. Depending on the route of administration, the agent can be coated with or in a material to protect it from the natural conditions which may detrimentally effect its ability to perform its intended function. The administration of the agent is done at dosages and for periods of time effective to significantly reduce or eliminate the symptoms associated with CF. Dosage regimes may be adjusted for purposes of improving the therapeutic response of the agent. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The language "effective amount" is intended to include that amount sufficient or necessary to significantly reduce or eliminate a subject's symptoms associated with CF.

The amount can be determined based on such factors as the type and severity of symptoms being treated, the weight and/or age of the subject, the previous medical history of the subject, and the selected route for administration of the agent. The determination of appropriate "effective amounts" is within the ordinary skill of the art. This definition applies throughout the present application for all agents including protein enhancing agents, differentiating agents and carboxy-compounds.

The present invention even further pertains to a method for treating a subject's lung epithelia containing a mutant CFTR protein. The method involves contacting a subject's lung epithelia with an agent which increases the level of the mutant CFTR at cellular locations where wild-type CFTR normally functions such that the mutant CFTR present in the lung epithelia mediates ion transport across the plasma membrane. The subject's lung epithelia can be contacted by administrating the agent to the subject. All other terms are as defined above.

The present invention also pertains to therapeutic compositions for treating a subject having CF. The composition contains a therapeutically effective amount of either a protein enhancing agent, a differentiating agent, and/or carboxy-compound, and a pharmaceutically acceptable carrier.

The language "therapeutically effective amount" is that amount sufficient or necessary to significantly reduce or eliminate a subject's symptoms associated with CF. The amount can vary depending on such factors as the severity of the symptoms being treated, the size of the subject, or the selected route for administration of the agent.

The language "pharmaceutically acceptable carrier" is intended to include substances capable of being co-administered with the agent and which allow the agent to perform its intended function, e.g. increasing the intracellular level of mutant CFTR at cellular locations where wild-type CFTR normally functions. Examples of such carriers include solvents, dispersion media, delay agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Any conventional media and agent compatible with the agent can be used with this invention. The agent of this invention can be administered alone or in a pharmaceutically accepted carrier. The agents further can be administrated as a mixture of agents which also can be in a pharmaceutically acceptable carrier. The agent further can be co-administered with other different art-recognized protein enhancing agents, differentiating agents, and/or adjuvants.

The present invention further pertains to a method for treating a subject having CF by administering an effective amount of a carboxy-compound to the subject. The carboxy-compound is administered such that a mutant CFTR present in CF-associated cells of the subject generates functional chloride channels or mediates chloride ion transport.

The present invention further pertains to a packaged drug for treating a subject having CF. The packaged drug includes a container holding an agent described above and instructions for administering the agent for treating a subject having CF. Examples of

containers include vials, syringes, etc. The instructions would contain dosage information for administering the agent as described above.

The present invention is further illustrated by the following examples which in no way should be construed as being further limiting. The contents of all cited references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference.

Example 1 - The Effect of Sodium Butyrate on Recombinant C127 Cells Expressing Δ F508 Cystic Fibrosis Transmembrane Conductance Regulator

Derivation of ΔF508-C127 Cells

A bovine-papilloma virus based eukaryotic expression vector (pBPV-CFTR-Δ F508) containing the gene for ΔF508 CFTR and neomycin resistance were transfected into C127 cells. The C127 cells are murine mammary cells which were obtained from ATCC (#CRL 1616). The expression of the mutant ΔF508 protein and neomycin was driven using a metallothionein promoter. Following transfection, clonal cells resistant to G418 were isolated and cells expressing the mutant ΔF508 protein were subsequently identified using antibodies specific for CFTR (mAb-13-1). The cells expressing the mutant ΔF508 CFTR protein were maintained in Dulbecco's modified eagle's media supplemented with glutamine and fetal calf serum.

Treatment of the ΔF508-C127 Cells with Butyrate and Analysis of Cells for Chloride Channel Activity

Recombinant C127 cells expressing ΔF508 CFTR were seeded onto glass coverslips. The cells were treated with butyrate (sold as sodium butyrate by Sigma Chemical, St. Louis, MO) (5mM to 50mM concentrating in cell growth medium) twenty-four hours after seeding for eighteen to twenty-four hours. After treatment with the butyrate, the cells were analyzed for the presence of cAMP-activatable chloride channels using the 6-methoxy-N-[3-sulfopropyl]-quinolinium (SPQ) assay. The cAMP-dependent chloride activity of the mutant CFTR was assessed using the halide-sensitive fluorophore SPQ. The cells were loaded with SPQ by including 10mM SPQ in the growth media for nine to twelve hours. The SPQ fluorescence was initially quenched by incubating the cells in a sodium iodide buffer solution (135mM NaI, 2.4mM K_2HPO_4; O.6mM KH_2PO_4; 1.0 mM $MgSO_4$; 1.0mM $CaSO_4$; 10.0mM HEPES pH 7.4). After measuring the fluorescence for two minutes using a Nikon inverted microscope, a Universal Imaging System and a Hamatsu camera, the sodium iodide buffer solution was replaced by a sodium nitrate buffer solution (same as the NaI solution except $NaNO_3$ was substituted for NaI). The fluorescence was measured for an additional 17.5 minutes. SPQ fluorescence is quenched by iodide but not by nitrate. The intracellular

cAMP levels were increased by adding forskolin and 3-isobutyl-1-methyl-xanthene (IBMX) five minutes after the anion substitution. In this assay (hereinafter the SPQ assay) an increase in halide permeability results in SPQ fluorescence.

As shown in Figure 1, the pretreatment of recombinant ΔF508-C127 cells with butyrate resulted in the generation of cAMP-dependent chloride channel activity. Approximately 80-90% of the cells exhibited a rapid increase in fluorescence following stimulation with forskolin and IBMX (which raises intracellular levels of cAMP). These cells therefore contained functional cAMP-dependent chloride channels. This activity was absent from ΔF508-C127 cells which had not been pretreated with butyrate and mock-transfected cells. Mock transfected cells are C127 cells which had been transfected with the bovine-papilloma-virus expression vector but not the ΔF508 mutant CFTR gene. These cells express the neomycin resistance gene product but not the mutant CFTR.

Example 2 - The Effect of Sodium Butyrate on Airway Epithelial Cells Derived from a Subject Having Cystic Fibrosis

Derivation of Airway Epithelial Cells

The human ΔF508 airway epithelial cells (DF) were derived from tissues of a CF patient homozygous for the ΔF508 mutation (gift from Dr. D. Jefferson, Tufts University, MA). The nasal epithelial cells were immortalized by SV40 Large-T antigen transduced using retroviruses. The DF cells were maintained in Dulbecco's modified eagle's media supplemented with adenine, insulin, transferrin, triodothyronine, epidermal growth factor and fetal calf serum.

Treatment of the Airway Epithelial Cells with Butyrate and Analysis of the Cells for Chloride Channel Activity

The DF cells were treated with butyrate and analyzed for chloride channel activity as described in Example 1 above. As shown in Figure 2, the pretreatment of DF cells with butyrate resulted in the generation of cAMP-dependent chloride channel activity. Approximately 10-20% of the cells displayed a measurable increase in fluorescence following induction by forskolin and IBMX. The smaller percentage of affected cells in this example probably reflected the lower level of expression of the ΔF508 CFTR protein in these cells (10-100 fold lower). The lower amount of ΔF508 CFTR protein in these cells may have precluded detection because of the limits of the sensitivity using this system.

Example 3 - The Effect of Temperature on Processing of CFTR ΔF508 and Wild-Type CFTR

3T3 fibroblasts expressing CFTR and CFTR ΔF508 were generated as described in Berger, H.A. et al. (1991) *J. Clin. Invest.* 88:1422-1431. Cells were seeded on

100 mm culture dishes and grown to 50-70% confluence. Cultures were then grown either at 37°C, 30°C, 26°C, or 23°C for two days. At the end of the incubation period, cultures were washed twice with phosphate-buffered saline and solubilized for thirty minutes at 4°C with lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.5, aprotinin at 1 μg/ml[2] and 1 mM PMSFP containing 1% recrystalized digitonin. Equal amounts of starting protein (1.2-1.4 mg per sample) were immunoprecipitated with monoclonal antibody mAb 13.1 and phosphorylated with (γ^{32}P) AIP and the catalytic subunit of PKA as described in Cheng, S.H. et al. (1990) *Cell* 83:827-834. Proteins were separated on 6% SDS-polyacrylamide gels and prepared for autoradiography. Quantitative assessment of radioactivity in gels and subtraction of background was done using a radioanalytic imaging system (AMBIS, San Diego, CA). To avoid problems inherent in attempts to quantify absolute amounts of protein by immunoprecipitation, data is presented as a percentage of total CFTR in band C. The results of this study, which are presented in Figure 3, indicate that percentage of mature CFTR produced from CFTR ΔF508 increases upon exposure to reduced temperatures.

Time-Dependence and Reversibility of Incubation at a Reduced Temperature

3T3 fibroblasts expressing CFTR ΔF508 were grown for 6, 12, 24, 48, 72, and 96 hours at 26°C. An autoradiograph of a 6% SDS polyacrylamide gel showed that as the length of incubation at 26°C was increased, the percent of CFTR ΔF508 present in the fully glycosylated form progressively increased.

3T3 fibroblasts expressing CFTR ΔF508 were grown for two days at 26°C and then grown at 37°C for 0 hours, 2 hours, 4 hours, 6 hours, 12 hours, or 24 hours. An autoradiograph of a 6% SDS polyacrylamide gel showed that the amount of mutant protein present as band C decreased with a half-life of about 7 hours.

Effect of Temperature on Processing Efficiency of CFTRΔF508

C127 cells (a cell line of mouse mammary epithelial origin) expressing CFTR or CFTRΔF508 were generated by calcium phosphate-mediated transfection with a bovine papilloma virus-based vector containing CFTR cDNA under control of the mouse metallathionein MT1 promoter and a neomycin-resistance gene (as the selectable marker) under the control of another copy of the MT1 promoter. Pulse-chase experiments were performed and assayed as described (Cheng. S.H. et al. *Cell* 83:827-834 (1990)), except that CDIM (carbon dioxide-independent medium; Gibco) was used for the chase, and was directed against a carboxy-terminal epitope. Under these conditions (100 μl of hybridoma supernatant and an overnight incubation) CFTR was maximally immunoadsorbed, so no additional CFTR was recovered after immunoprecipitation with monoclonal antibody mAb13-1 following immunoprecipitation with mAb 24-1.

C127 cells expressing CFTRΔF508 were grown for 40 hours, at 26°C, pulsed with 360 μCi[^{36}S] methionine per ml. for 15 min. at 37°C, then chased at 37°C or 26°C after

4 hours and 5 hours. Immunoprecipitates of cell lysates were separated on a 6% SDS-polyacrylamide gel, fluorographed with salicylate and visualized by autoradiography. The processing efficiency of CFTR in C127 cells was unaffected by temperature. In contrast, the results of these experiments show that mutant protein synthesized at 37°C is processed normally during subsequent incubation at 26°C but not at 37°C.

Effect of Temperature on cAMP-regulated Cl^- Channel Current

Whole-cell and single-channel patch-clamp studies were done as described (Rich, D.P. et al. (1991) *Science* 253:202-205). For whole-cell studies the pipette (internal solution) contained (in mM) 120 N-methyl-D-glucamine (NMDG), 5 HEPES, 3 $MgCl_2$ and 1 Cs-EGTA (adjusted to pH 7.3 with HCl). The bath contained 140 NaCl, 10 HEPES, 1.2 $CaSO_4$ 1.2$MgSO_4$ 10 dextrose (to pH 7.3 with NaOH). When bath Cl^- concentration was reduced, 120 sodium aspartate replaced 120 NaCl. For studies in excised inside-out patches, the pipette (external) solution contained (in mM):140 NMDG, 2 $MgCl_2$, 5 $CaCl_2$, 100 L-aspartic acid, and 10 HEPES (to pH 7.3 with HCl) (Cl^- concentration, 49 mM). The bath (internal) solution contained (in mM): 140 NMDG, 3 $MgCl_2$, 1 Cs EGTA, and 10 HEPES (to pH 7.3 with HCl) (Cl^- concentration, 147 mM). All experiments were done at 35°C. Open-state probability (P_o) and single-channel conductance were determined from amptitude histograms, P_o was measured in patches containing 5 channels. The number of channels was determined from the maximum number simultaneously open with 3 mM ATP.

3T3 fibroblasts and C127 cells expressing CFTRAUG 26, 1992F508 were cultured for 2 days at 30°C or 37°C. One group of 3T3 fibroblasts were incubated at 30°C for 2 days and then at 37°C for 1 day. cAMP was increased by addition of 250 μM 8-(4-chloreophenylthio) adenosine cyclic monophosphate (CPT-cAMP) and 20 μM forskolin.

The number of cells studied in each group and the cell capacitance was 3T3 cells at 37°C, 27.2±1.2 pF(n=12); 3T3 cells at 30°C, 30.4±3.0 pF(n=10); 3T3 cells 30°C, and then 37°C, 31.1-+8.8 (n=5); C127 cells at 37°C, 27.6±2.4 (n=11); C127 cells at 30°C, 30.2±3.8 (n=10). The cAMP-stimulated current was greater in cells cultured at 30°C than at 37°C for both 3T3 and C127 cells ($P<0.012$). Current-voltage relationship of whole-cell currents under basal conditions after stimulation with cAMP agonists, and when the Cl^- concentration in the bathing solution was reduced from 140 mM to 22mM by substitution of Cl^- with aspartate, low Cl^- current-voltage relationships were obtained by a ramp of voltage from -40mV to +40mV. Representative whole-cell currents were generated by voltage steps from -100 mV to +100 mV in 50-mV increments; holding voltage was -40mV. As an example of CFTR Cl^- channels in an excised, inside-out patch of membrane from 3T3 fibroblasts expressing CFTRΔF508, the cell was incubated at 30°C for 2 days. Tracings were obtained under basal conditions, in the presence of 1 nM ATP, in the presence of 1 nM ATP plus 75 nM catalytic subunit of PKA, and after ATP and PKA were removed (wash). All

traces were obtained at -100mV. The patch contained multiple channels. All patch-clamp studies were performed at 35°C.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

Claims

1. A method for generating cystic fibrosis transmembrane conductance regulator function in a cell containing mutant cystic fibrosis transmembrane conductance regulator, comprising:
contacting the cell with an agent which increases the level of the mutant cystic fibrosis transmembrane conductance regulator at cellular locations where wild-type cystic fibrosis transmembrane conductance regulator normally functions such that the mutant cystic fibrosis transmembrane conductance regulator generates functional ion channels or mediates ion transport at the cellular locations where wild-type cystic fibrosis transmembrane conductance regulator normally functions.

2. The method of claim 1 wherein the agent is a protein enhancing agent.

3. The method of claim 1 wherein the agent is a mobilizing agent.

4. The method of claim 1 wherein the agent is a differentiating agent.

5. The method of claim 1 wherein the agent is a carboxy-compund.

6. The method of claim 1 wherein the cells are airway epithelia.

7. The method of claim 1 wherein a cellular location where wild-type cystic fibrosis transmembrane conductance regulator normally functions is the plasma membrane.

8. A method for generating cystic fibrosis transmembrane conductance regulator function in an airway epithelial cell containing mutant cystic fibrosis transmembrane conductance regulator, comprising:
contacting the airway epithelial cell with an agent which increases the level of the mutant cystic fibrosis transmembrane conductance regulator at the plasma membrane such that the mutant cystic fibrosis transmembrane conductance regulator mediates ion transport across the plasma membrane.

9. The method of claim 8 wherein the agent is a protein enhancing agent.

10. The method of claim 8 wherein the agent is a mobilizing agent.

11. The method of claim 8 wherein the agent is a differentiating agent.

12. The method of claim 8 wherein the agent is a carboxy-compound.

13. A method for redistributing subcellular locations of mutant cystic fibrosis transmembrane conductance regulator in a cell containing mutant cystic fibrosis transmembrane conductance regulator, comprising:
contacting the cell with an agent which increases the level of the mutant cystic fibrosis transmembrane conductance regulator at cellular membranes across which wild-type cystic fibrosis transmembrane conductance regulator normally mediates ion transport such that the mutant cystic fibrosis transmembrane conductance regulator mediates ion transport across the cellular membranes which wild-type cystic fibrosis transmembrane conductance regulator normally mediates ion transport.

14. The method of claim 16 wherein the agent is a protein enhancing agent.

15. The method of claim 13 wherein the agent is a mobilizing agent.

16. The method of claim 13 wherein the agent is a differentiating agent.

17. The method of claim 13 wherein the agent is a carboxy-compound.

18. A method for treating a subject having cystic fibrosis, comprising:
administering to the subject in an effective amount of an agent which increases the level of mutant cystic fibrosis transmembrane conductance regulator at cellular locations where wild-type cystic fibrosis transmembrane conductance regulator normally functions such that the mutant cystic fibrosis transmembrane conductance regulator generates functional ion channels at the cellular locations where wild-type cystic fibrosis transmembrane conductance regulator normally functions.

19. The method of claim 18 wherein the agent is a protein enhancing agent.

20. The method of claim 18 wherein the agent is a mobilizing agent.

21. The method of claim 18 wherein the agent is a differentiating agent.

22. The method of claim 16 wherein the agent is a carboxy-compound.

23 The method of claim 18 wherein the cellular location where the level of mutant cystic fibrosis transmembrane conductance regulator increases is the plasma membrane.

24. A method for treating a subject's lung epithelia containing a mutant cystic fibrosis transmembrane conductance regulator, comprising:

contacting a subject's lung epithelia with an agent which increases the level of mutant cystic fibrosis transmembrane conductance regulator at the plasma membrane of the lung epithelial cells such that the mutant cystic fibrosis transmembrane conductance regulator present in the plasma membrane mediates ion transport across the plasma membrane.

25. The method of claim 30 wherein the agent is a protein enhancing agent.

26. The method of claim 24 wherein the agent is a mobilizing agent.

27. The method of claim 24 wherein the agent is a differentiating agent.

28. The method of claim 24 wherein the agent is a carboxy-compund.

29. A therapeutic composition for treating a subject having cystic fibrosis, comprising:

a therapeutically effective amount of an agent which increases the level of mutant cystic fibrosis transmembrane regulater at cellular locations within a subject where wild type cystic fibrosis transmembrane conductance regulator normally functions; and

a pharmaceutically acceptable carrier.

30. The therapeutic composition of claim 29 wherein the agent is a protein enhancing agent.

31. The therapeutic composition of claim 29 wherein the agent is a mobilizing agent.

32. The therapeutic composition of claim 29 wherein the agent is a differentiating agent.

33. The therapeutic composition of claim 29 wherein the agent is a carboxy-compound.

34. A packaged drug for treating a subject having cystic fibrosis, comprising:
a container holding an agent which increases the level of mutant cystic fibrosis transmembrane regulater at cellular locations within a subject where wild type cystic fibrosis transmembrane conductance regulator normally functions; and
instructions for administering the agent for treating a subject having cystic fibrosis.

35. The therapeutic composition of claim 34 wherein the agent is a protein enhancing agent.

36. The thereapeutic composition of claim 34 wherein the agent is a mobilizing agent.

37. The therapeutic composition of claim 34 wherein the agent is a differentiating agent.

38. The therapeutic composition of claim 34 wherein the agent is a carboxy-compound.

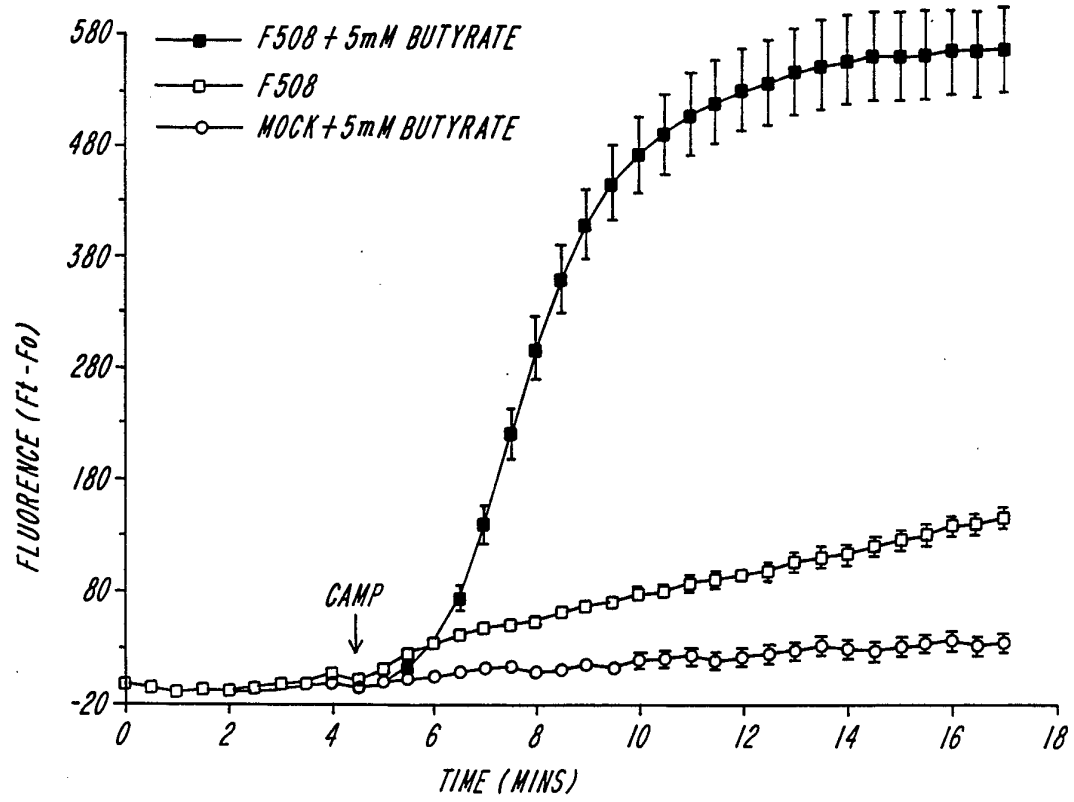

F508 + 5mM BUTYRATE
F508
MOCK + 5mM BUTYRATE
FLUORENCE (Ft-Fo)
580
480
380
280
180
80
-20
CAMP
0
2
4
6
8
10
12
14
16
18
TIME (MINS)

FIG. 1

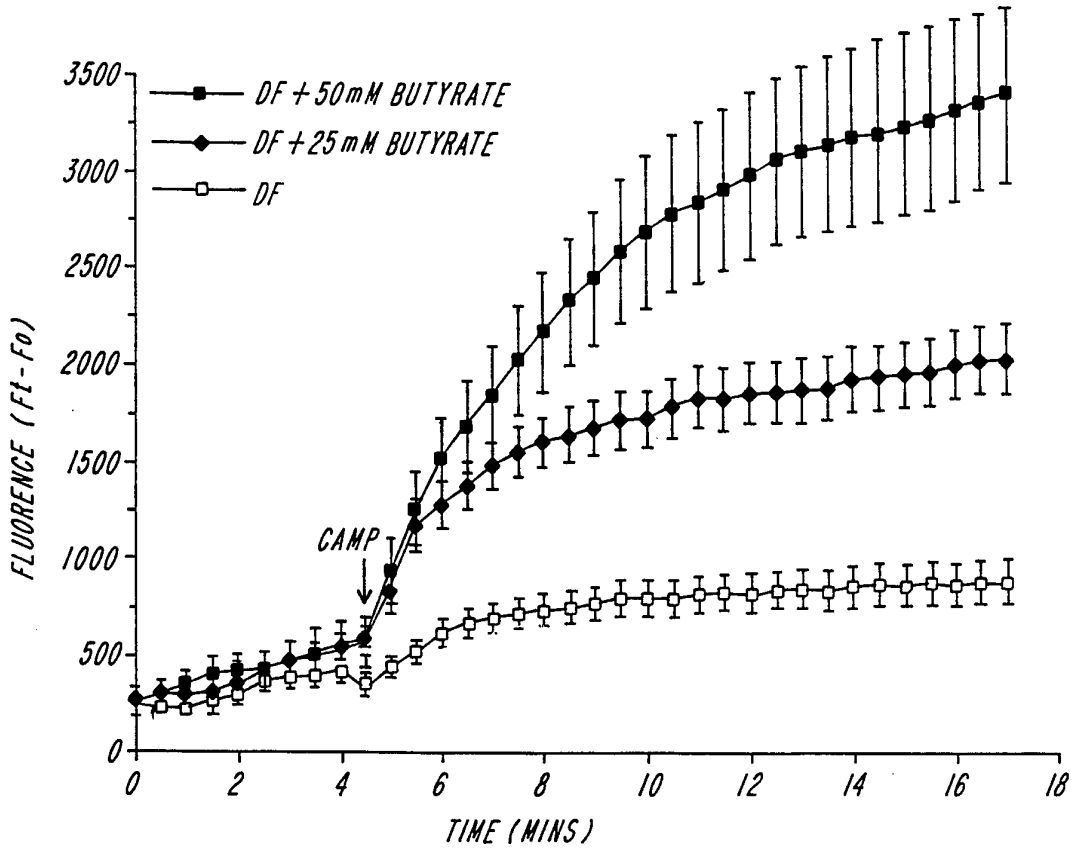

DF + 50mM BUTYRATE
DF + 25mM BUTYRATE
DF
FLUORENCE (Ft-Fo)
3500
3000
2500
2000
1500
1000
500
0
CAMP
0
2
4
6
8
10
12
14
16
18
TIME (MINS)

FIG. 2

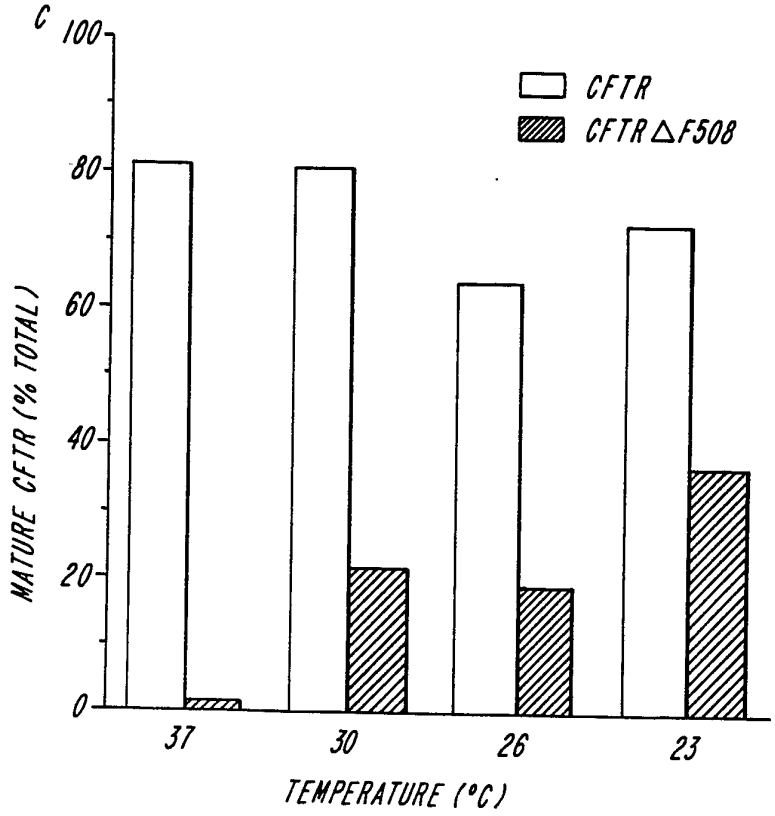

C
100
80
60
40
20
0
MATURE CFTR (% TOTAL)
CFTR
CFTR ΔF508
37
30
26
23
TEMPERATURE (°C)

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/08028

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 15/00, 5/00; C12P 21/06; C07K 13/00
US CL :435/320.1, 240.1, 69.1; 530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1, 240.1, 69.1; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Science, Vol. 254, issued December 1991, M.L. Drumm et al., "Chloride conductance expressed by deltaF508 and other mutant CFTRs in Xenopus oocytes", pages 1797-1799.	1-38
A	J. Biol. Chem., Vol. 263, No. 12, issued 25 April 1988, C.E. Machamer et al., "Vesicular stomatitis virus G proteins with altered glycosylation sites display temperature-sensitive intracellular transport and are subject to aberrant intermolecular disulfide bonding", pages 5955-5960.	1-38

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
17 November 1993

Date of mailing of the international search report
03 DEC 1993

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